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The EIIIA Segment of Fibronectin Is a Ligand for Integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ Providing a Novel Mechanism for Regulating Cell Adhesion by Alternative Splicing*

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Alternative splicing of the fibronectin gene transcript gives rise to forms that include the EIIIA (or ED-A) segment. EIIIA-containing fibronectins are prominently expressed during embryogenesis and wound healing and appear to mediate changes in cell adhesion and gene expression. Nonetheless, integrins that bind the EIIIA segment have not been identified. We previously mapped the epitope for two function-blocking monoclonal antibodies to the C-C′ loop region of the EIIIA segment (Liao, Y.-F., Wieder, K. G., Classen, J. M., and Van De Water, L. (1999) *J. Biol. Chem.* 274, 17876–17884). The sequence of this epitope (^{39}PEDGIHELFP^{48}) resembles the sequence within tenascin-C to which the integrin $\alpha_9\beta_1$ binds. We now report that either integrin $\alpha_9\beta_1$ or $\alpha_4\beta_1$ can mediate cell adhesion to the EIIIA segment. Moreover, this interaction is blocked both by epitope-mapped EIIIA antibodies as well as by the respective anti-integrins. Deletion mutants of the EIIIA segment that include the C-C′ loop and flanking sequence bind cells expressing either $\alpha_9\beta_1$ or $\alpha_4\beta_1$. Adhesion of $\alpha_4\beta_1$-containing MOLT-3 cells to the EIIIA segment stimulates phosphorylation of p44/42 MAP kinase. Our observation that two integrins bind the EIIIA segment establishes a novel mechanism by which cell adhesion to fibronectin is regulated by alternative splicing.

Although it has been clear for many years that fibronectin (FN)[1] is alternatively spliced, the functions of, and receptors for, two alternatively spliced segments termed EIIIA (or ED-A) and EIIIB (or ED-B) segments have remained elusive. More is known about a non-homologous IIICS repeat encoding the CS-1 segment, which is a cell adhesive site and ligand for integrin $\alpha_4\beta_1$ (1). Both the EIIIA and EIIIB segments are homologous FN type III repeats and are prominently expressed during embryogenesis; homozygous mutations in FN are embryonic lethal (2–7). During wound healing (5, 8), lung, liver, and kidney fibrosis (9–11), vascular intimal proliferation (12, 13), vascular hypertension (14), and cardiac transplantation (15), the expression of FNs containing the EIIIA and EIIIB domains is significantly increased. A ~170-kDa species of EIIIA-containing FNs is found in synovial fluid from patients with rheumatoid arthritis but not osteoarthritis (16). The EIIIB segment has been postulated to have a role in angiogenesis (17). The EIIIA segment has been observed to regulate cell adhesion and proliferation (18–21). Liver lipocytes and skin fibroblasts differentiate into myofibroblasts when adhering to FNs that include the EIIIA segment (10, 22). One monoclonal antibody (IST-9) to the EIIIA segment has been shown to inhibit myofibroblast differentiation, whereas another (DH1) blocks chondrogenesis during chick development (10, 22, 23). Moreover, the expression of MMP-9 is regulated by the EIIIA segment in chondrocytes and myelomonocytic cells potentially through toll-like receptors (24, 25).

We recently reported detailed epitope maps for function-blocking monoclonal antibodies that bind to the C-C′ loop of the EIIIA segment (26). The FN type III (FN-III) repeats, of which the EIIIA segment is one, exhibit high structural homology (27–31) despite only 20–40% identity in amino acid sequence (32). The canonical FN type III repeat is a conserved β-sandwich conformation consisting of two β sheets comprising four strands (G, F, C, C′) and three strands (A, B, and E) (27). Epitope mapping of the EIIIA segment reveals that function-blocking mAbs interact with the loop between the C and C′ β-strands and the adjacent Ile^{43} and His^{44} residues are critical to the epitope (26). Given that these monoclonal antibodies blocked EIIIA function we reasoned that the peptide comprising the C-C′ loop region (EDGIHEL) could encode a sequence that bound cell surface receptors, possibly integrins.

The integrins are a family of heterodimeric transmembrane receptors that mediate cell-extracellular matrix and cell-cell interactions (33). One integrin, $\alpha_9\beta_1$, binds to a peptide sequence within the B-C loop of tenascin-C (34). This sequence (AEIDGIEL) is similar to the EDGIHEL sequence that we identified in the EIIIA segment (26). The α_9 subunit binds unrelated sequences in other ligands including the vascular cell adhesion molecule-1 (VCAM-1) (35), osteopontin (36), the propolypeptide of von Willebrand factor (pp-vWF) (37), tissue transglutaminase (tTG) (37), blood coagulation factor XIII (FXIII) (37), and L1-CAM (38). These ligands, with the exception of tenascin-C, also bind to integrin $\alpha_4\beta_1$, the closest relative of $\alpha_9\beta_1$ with which it shares 39% amino acid identity (37,

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[1] The abbreviations used are: FN, fibronectin; FN-III, fibronectin type III repeat; MAP, mitogen-activated protein; mAbs, monoclonal antibodies; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GST, glutathione *S*-transferase; MMP-9, matrix metalloprotease-9.

39–42). We now report that the full-length EIIIA segment and deletion mutants that include the C-C′ loop region of the EIIIA segment serve as ligands for integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ on cells.

MATERIALS AND METHODS

Reagents—The anti-EIIIA mAbs, IST-9 (43) and 3E2, were purchased from Harlan Bioproducts (Indianapolis, IN) and Sigma, respectively. Anti-human integrin $\alpha_9\beta_1$ (clone Y9A2) was prepared as previously described (44). Mouse mAbs against human integrins α_4 (clone P4C2) and α_5 (clone P1D6) were purchased from Invitrogen. Another mouse mAb against human integrin α_4 (clone HP2/1) was purchased from Beckman Coulter, Inc. (Fullerton, CA). FITC-conjugated goat anti-mouse IgG and mouse IgG1 were purchased from Zymed Laboratories Inc.. Mouse anti-β_1 integrin mAb (clone P4C10) was a gift from Dr. Donald Senger (Beth Israel Deaconess Medical Center). Histidine-tagged recombinant FN III repeats were prepared and purified as previously described (45). Bovine thrombin and AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) hydrochloride were from Calbiochem. BCA protein assay reagent kit and Reacti-Bind maleic anhydride-activated polystyrene 96-well plates were purchased from Pierce. Tissue culture media and fetal bovine serum were purchased from Invitrogen. Neomycin analog, G418, glutathione-agarose, and serum replacement medium SITE+3 were from Sigma. Complete™ protease inhibitor mixture was from Roche Molecular Biochemicals. Rabbit anti-phospho-p44/42 MAP kinase antibody, anti-p44/42 MAP kinase antibody, and horseradish peroxidase-conjugated anti-rabbit IgG were from Cell Signaling Technology, Inc. (Beverly, MA). All other reagents were at least reagent grade and obtained from standard suppliers.

Cell Culture—Integrin α_9- or mock-transfected SW480 human colon cancer cells (SW-α_9 or SW-mock) were generated as described (46) and maintained in DMEM supplemented with 1 mg/ml G418, 10% fetal bovine serum, and 0.1 mg/ml penicillin and streptomycin. MOLT-3 human acute lymphoblastic leukemia cells were purchased from American Type Culture Collection and cultured in RPMI 1640 containing 10% fetal bovine serum. Cells were incubated in a humidified incubator at 37 °C in 10% CO_2.

Cell Adhesion Assays—Cell adhesion assays were performed as previously described (47). Soluble recombinant FN III repeats (10 μg/ml in PBS) were coated on the wells of 96-well flat-bottomed microtiter plates (Corning-Costar) at 4 °C overnight. Wells were washed with PBS and blocked by 1% BSA in DMEM (for SW480) or RPMI 1640 (for MOLT-3) at 37 °C for 1 h. SW480 cells were detached using EDTA (20 mM in PBS), washed, and resuspended in serum-free DMEM. MOLT-3 cells were harvested by centrifugation and resuspended in serum-free RPMI 1640 containing 250 μM $MnCl_2$. For blocking experiments, cells were preincubated either with Y9A2 (10 μg/ml) or P4C2 (10 μg/ml) at 4 °C for 15 min or with various concentrations of synthetic peptides for 30 min at 4 °C before plating. Cell suspensions (100 μl/well of 50,000 cells/ml in serum-free medium with 0.5% BSA) either with or without pretreatments were then plated directly into wells. Plates were centrifuged (top side up) at 10 × *g* for 5 min followed by incubation for 1 h (for SW480) or 90 min (for MOLT-3) at 37 °C in a humidified incubator with 5% CO_2. Nonadherent cells were removed by centrifugation (top side down) at 48 × *g* for 5 min. Adherent cells were fixed and stained with crystal violet (0.5% w/v in 1% formaldehyde and 20% methanol) for 1 h at room temperature followed by washes with PBS. Stained cells were dissolved by 2% Triton X-100 in PBS, and the absorbance at 570 nm was determined in a ThermoMax microplate reader (Molecular Devices, CA). Experiments were conducted in triplicate and included BSA-coated wells as a blank. When EIIIA-specific mAbs were employed as the competitors for cell adhesion, EIIIA-coated wells were pretreated with various dilutions of these mAbs as denoted in figure legends. Unbound antibodies were removed by washes with PBS prior to the addition of cells.

Alternatively, the EIIIA proteins (1 μM in PBS) were covalently linked to maleic anhydride Reacti-Bind microplates at 4 °C overnight. Protein-coated wells were washed with PBS and blocked with 1% BSA in PBS at 37 °C for 1 h. Cells were harvested and resuspended in Hanks' buffered salt solution (HBSS) (10^6 cells/ml) and labeled with 2 μM BCECF-AM (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluoresceintetrakis(acetoxymethyl) ester) at room temperature for 30 min. Labeled cells were then washed with serum-free DMEM and resuspended in 0.5% BSA-DMEM. Prior to plating, MOLT-3 cells were pretreated with 250 μM $MnCl_2$ on ice for 30 min. Cells (5 × 10^4 cells/well) were allowed to adhere to the coated wells at 37 °C for 60 min (for SW480 cells) or 90 min (for MOLT-3 cells). Nonadherent cells were removed by centrifugation (top side down) at 48 × *g*. Adherent cells were resuspended in 200 μl of DMEM (SW480 cells) or RPMI 1640 (MOLT-3 cells), and fluorescence was quantified with a fluorometric plate reader (Molecular Devices) at excitation wavelength 485 nm and emission wavelength 538 nm.

Thrombin Cleavage of the GST-tagged EIIIA Segment—GST-tagged wild type and deletion mutants of the EIIIA segment were purified as previously described (26). Proteins were re-attached to 200 μl of glutathione-agarose (50% slurry) in microfuge tubes at 4 °C for 1 h with gentle agitation, followed by three washes with PBS. Bovine thrombin (100 μl of 100 unit/ml) was added to protein-attached agarose beads and incubated at room temperature for 2–4 h. Cleaved EIIIA segments were separated from glutathione-agarose beads by centrifugation, and the reaction was stopped by the addition of 10 μl of AEBSF (100 mM). The purity of the cleaved EIIIA segments was examined by SDS-PAGE (data not shown). Protein concentrations of the cleaved products were quantified by the BCA protein assay reagent kit.

MAP Kinase Activation of MOLT-3 Cells—To test MAP kinase activation, MOLT-3 cells were cultured in RPMI 1640 supplemented with SITE+3 serum replacement medium for 3 days. Anti-α_4 integrin mAb (HP2/1, 2 μg/ml) and histidine-tagged EIIIA segment (100 μg/ml) were coated onto 6-well plates (1 ml/well) at 4 °C overnight. On the day of the experiment, MOLT-3 cells were washed with RPMI 1640 and resuspended in RPMI 1640,0.5% BSA at 2 × 10^6 cells/ml. This was followed by pretreatment with 250 μM $MnCl_2$ at 4 °C for 30 min prior to plating. Protein-coated wells were blocked with 1% BSA/PBS at 37 °C for 1 h and washed with PBS. Mn^{2+}-treated MOLT-3 cells were layered onto the protein-coated wells, allowed to settle at 4 °C for 30 min, and then brought to 37 °C for the time specified before being placed on ice. After the reactions were terminated, the medium was aspirated and nonadherent cells were removed by brief centrifugation (plate inverted) at 48 × *g*. Adherent cells were lysed in ice-cold cell extraction buffer containing 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, and Complete™ protease inhibitor mixture. Cell lysates were scraped off the plates and transferred to microfuge tubes. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min following extensive vortexing. Postnuclear supernatants were collected and analyzed by Western blotting as described below.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Clarified cell extracts were mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% dithiothreitol, and 5% β-mercaptoethanol) and boiled at 100 °C for 5 min. Samples were then analyzed by Western blotting (26). Proteins were resolved in precast Tris glycine polyacrylamide gels (4–20%) (Invitrogen) in duplicate. Separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Bio-Rad) overnight at 4 °C. Membranes were treated with blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in PBS) at room temperature for 2 h. Following a brief rinse, one of the duplicate membranes was probed with an anti-phospho-p44/42 MAP kinase polyclonal antibody, and the other was probed with an anti-p44/42 MAP kinase polyclonal antibody (1:1000 dilution in SuperBlock (Pierce), respectively) for 2 h at room temperature. This was followed by washes in PBST (0.05% Tween 20 in PBS). Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 in SuperBlock) at room temperature for 1 h. Following three washes in PBST, the immunoblots were then incubated with Supersignal (Pierce) chemiluminescence substrate for 5 min and exposed to a phosphor cassette. Images of the blots were processed with the Molecular Image System GS-525 using Multi-Analysis software version 1.1 (Bio-Rad).

RESULTS

Integrin $\alpha_9\beta_1$ Mediates Cell Adhesion to the EIIIA Segment of FN—Sequence comparisons revealed that the C-C′ loop region within the EIIIA segment resembled the ligand binding site for integrin $\alpha_9\beta_1$ in the third FN-III repeat of tenascin-C. This finding prompted us to determine whether or not the EIIIA segment could be a novel ligand for integrin $\alpha_9\beta_1$. We conducted cell adhesion assays with SW480 cells that had been stably transfected with either an α_9-expression plasmid (SW-α_9) or empty vector (SW-mock) (46). Wells were coated with recombinant fusion proteins representing either the EIIIA segment alone or the fourth type III repeat, FN-III4, alone. Cells were then allowed to adhere to coated wells either in the presence or absence of Mn^{2+}, and the specificity of $\alpha_9\beta_1$-medi-

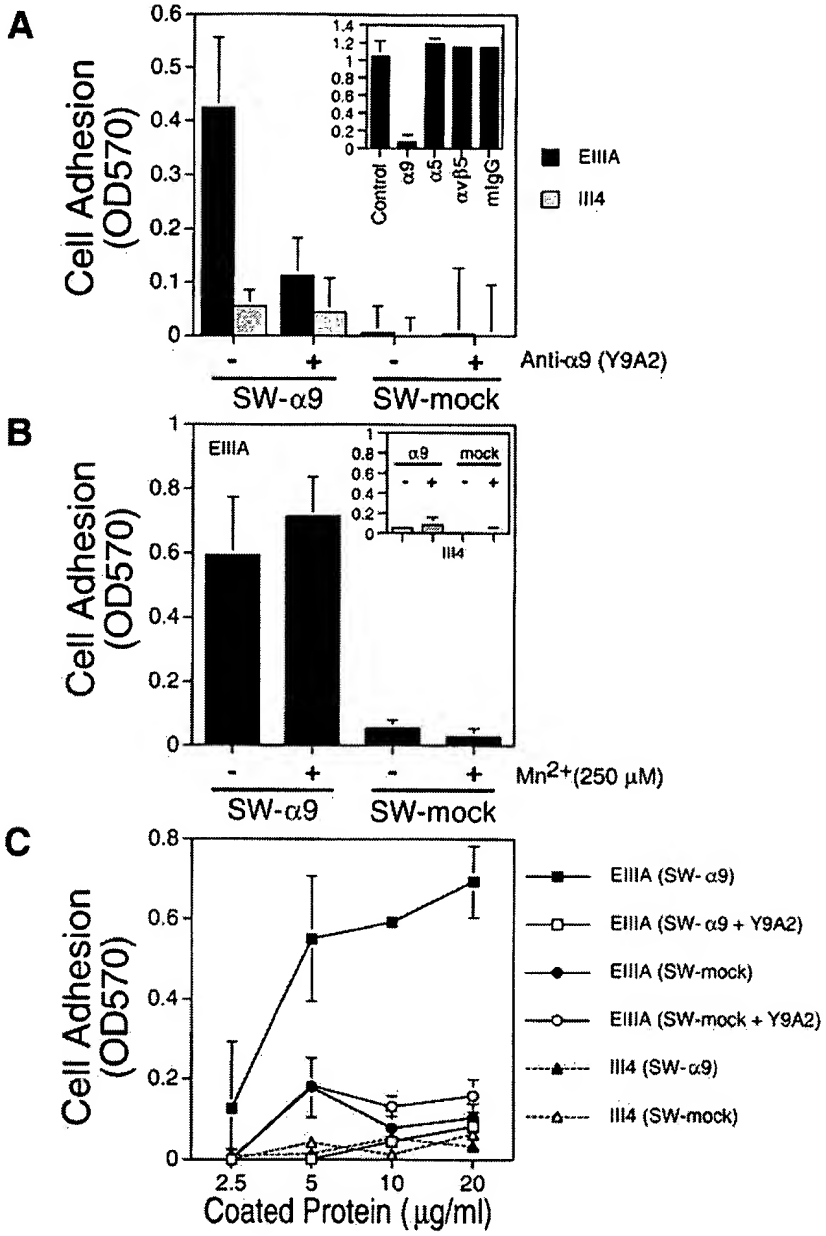


FIG. 1. **Adhesion assays using either α9- (SW-α9) or mock-transfected (SW-mock) SW480 cells.** *A*, integrin $\alpha_9\beta_1$ specifically adheres to the EIIIA segment. Recombinant histidine-tagged EIIIA and FN-III4 (10 μg/ml in PBS) were coated onto 96-well microtiter plates overnight at 4 °C. Transfected SW480 cells were preincubated with or without anti-α_9 blocking antibody Y9A2 (10 μg/ml) for 30 min at 4 °C before plating. *Solid bar*, the adhesion to EIIIA-coated wells; *shaded bar*, the adhesion to FN-III4 coated wells; +, pretreatment with Y9A2; −, no treatment. *Inset*, an independent experiment using blocking antibodies to integrins α_9 (Y9A2, 10 μg/ml), α_5 (P1D6, 10 μg/ml), or $\alpha_v\beta_5$ (P1F6, 10 μg/ml) shows the adhesion of SW-α_9 to EIIIA does not involve other β_1-associated integrins or non-β_1 integrins. Mouse IgG (mIgG, 10 μg/ml) was used as a control. *B*, Mn^{2+} is not required for the adhesion of SW-α_9 cells to the EIIIA segment. Recombinant histidine-tagged EIIIA (10 μg/ml in PBS) were coated onto 96-well microtiter plates overnight at 4 °C. Cells were pretreated with or without Mn^{2+} (250 μM) at 4 °C for 30 min before plating. *Inset*, a separate experiment indicating that the presence of Mn^{2+} does not promote the adhesion of SW-α_9 cells to FN-III4 (10 μg/ml in PBS) (*shaded bar*). +, pretreatment with Mn^{2+}; −, no treatment. *C*, SW-α_9 cells adhere to the EIIIA segment in a dose-dependent manner. Various concentrations of recombinant histidine-tagged EIIIA and FN-III4 were coated onto 96-well microtiter plates overnight at 4 °C. SW480 cells were preincubated with or without Y9A2 (10 μg/ml) for 30 min at 4 °C before plating. Key at *right* shows different permutations of coated proteins, cell lines, and treatments. For all these experiments, cells were allowed to attach to protein-coated wells at 37 °C for 1 h, and nonadherent cells were removed by centrifugation as described under "Materials and Methods." Adherent cells were stained with crystal violet and quantified by measurement of absorbance at 570 nm. Results from a representative experiment are expressed as the mean (± S.D.) of triplicate measurements.

ated adhesion was evaluated by an $\alpha_9\beta_1$-blocking mAb, Y9A2. We found that the SW-α_9 cells exhibited significant adhesion to the EIIIA segment but not to FN-III4, and this adhesion was blocked by Y9A2 (Fig. 1*A*). Moreover, this inhibition was specific for $\alpha_9\beta_1$. Neither anti-α_5 nor anti-$\alpha_v\beta_5$ blocking antibody inhibited this adhesion (Fig. 1*A*, *inset*), and SW-mock transfected cells that express native $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ did not adhere to EIIIA-coated wells (Fig. 1*B*) or FN-III4 (*inset*). Pretreatment with Mn^{2+} did not significantly enhance the adhesion of either SW-mock or SW-α_9 cells to EIIIA (Fig. 1*B*) or to FN-III4 (*inset*). The α_9-mediated adhesion to EIIIA was strictly dependent on the concentration of EIIIA used to coat the wells (Fig. 1*C*).

Integrin $\alpha_4\beta_1$ Mediates Adhesion of MOLT-3 Cells to EIIIA Segment—A number of ligands for $\alpha_9\beta_1$, including osteopontin, vascular cell adhesion molecule-1 (VCAM-1), the propolypeptide of von Willebrand factor (pp-vWF), tissue transglutaminase (tTG), and blood coagulation factor XIII (FXIII), have also been observed to bind the closely related integrin $\alpha_4\beta_1$. Because the EIIIA segment bound α_9-transfected cells, we sought to determine whether or not $\alpha_4\beta_1$ also served as a receptor for the EIIIA segment. MOLT-3 cells were used because of their significant expression of $\alpha_4\beta_1$ and their lack of $\alpha_9\beta_1$ (37). Mn^{2+}-pretreated MOLT-3 cells significantly adhered to the EIIIA segment but only minimally to FN-III4. MOLT-3 cells without the pretreatment of Mn^{2+} did not adhere to either FN segment (Fig. 2*A*). The adhesion of Mn^{2+}-treated MOLT-3 cells to the EIIIA segment was blocked by an anti-α_4 mAb, P4C2 (Fig. 2*A*). Complete inhibition of MOLT-3 cells adhesion to the EIIIA segment was observed with either anti-α_4 and anti-β_1 blocking antibodies or with CS-1 peptide, a specific ligand for integrin α_4 (Fig. 2*B*), indicating that this adhesion was specifically mediated by integrin $\alpha_4\beta_1$ rather than other β_1 integrins.

Function-blocking Anti-EIIIA Antibodies Block $\alpha_9\beta_1$- and $\alpha_4\beta_1$-mediated Binding—Several mAbs to EIIIA have been shown to block the differentiation of fibroblasts into myofibro-

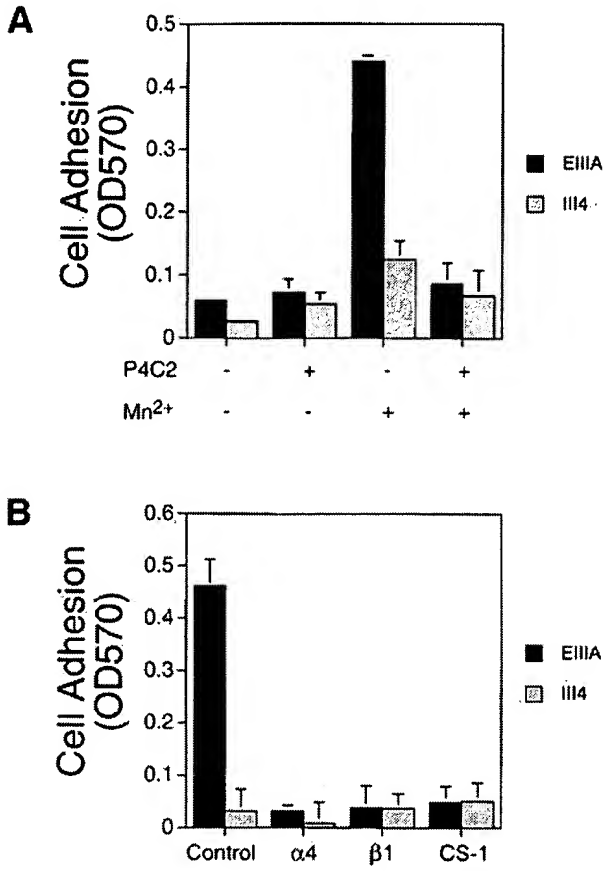


FIG. 2. **Adhesion assays using MOLT-3 cells that express integrin $\alpha_4\beta_1$.** *A*, integrin a4b1 specifically adheres to the EIIIA segment in the presence of Mn^{2+}. Recombinant EIIIA (*solid bar*) or FN-III4 (*shaded bar*) (10 μg/ml in PBS) were coated onto 96-well microtiter plates overnight at 4 °C. MOLT-3 cells were either untreated or pretreated with Mn^{2+} (250 μM) at 4 °C for 30 min followed by incubations in the presence or absence of anti-α_4 blocking antibody (P4C2, 10 μg/ml) at 4 °C for 30 min prior to plating. *B*, integrin $\alpha_4\beta_1$ is the only β_1 integrin expressed by MOLT-3 cells that adheres to the EIIIA segment. Recombinant EIIIA (*solid bar*) or FN-III4 (*shaded bar*) (10 μg/ml in PBS) were coated onto 96-well microtiter plates overnight at 4 °C. MOLT-3 cells were pretreated with Mn^{2+} (250 μM) at 4 °C for 30 min followed by incubations in the absence (*Control*) or presence of anti-α_4 (HP2/1, 10 μg/ml), anti-β_1 (P4C10, 10 μg/ml) antibodies, or CS-1 peptide (BSA-conjugated, 2 μg/ml) at 4 °C for 30 min prior to plating. Treated MOLT-3 cells were added to wells coated with either EIIIA or FN-III4 and incubated at 37 °C for 90 min. Attached cells were quantified by measurements of absorbance at 570 nm. Results were shown as the mean (± S.D.) of triplicate measurements. Various treatments are shown at the bottom. +, pretreatment with Mn^{2+} or P4C2; −, no treatment.

blasts as well as the process of chondrogenesis (see Introduction). The epitopes for these EIIIA-specific mAbs reside in the C-C′ loop of EIIIA (26). To examine whether and to what extent these mAbs blocked $\alpha_9\beta_1$- and $\alpha_4\beta_1$-mediated cell attachment to the EIIIA segment, we preincubated the EIIIA segment with either monoclonal antibodies IST-9 or 3E2. We observed a dramatic inhibition of the adhesion of either SW-α_9 or MOLT-3 to the EIIIA segment (Fig. 3). These data provide further support for a specific interaction between the EIIIA segment and integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$. These results also raise the possibility that the ligand binding sites within EIIIA for $\alpha_9\beta_1$ and $\alpha_4\beta_1$ include the C-C′ loop region.

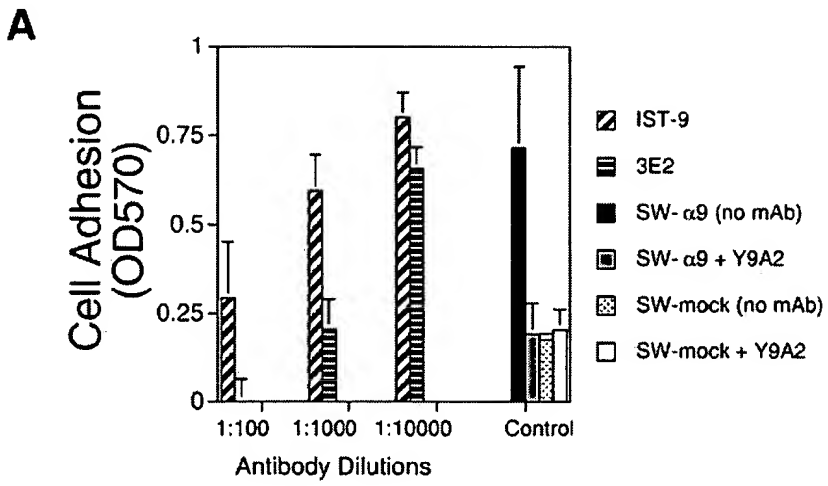
Integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ Bind to Deletion Constructs of the EIIIA Segment—To define further the sequences within the EIIIA segment essential for the binding of integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$, we tested whether and to what extent these integrins mediated cell binding to a panel of EIIIA deletion mutants previously generated (26) (Fig. 4). These deletion mutants of the EIIIA segment were generated as GST-tagged recombinant proteins. Their GST tags were subsequently removed by thrombin cleavage to eliminate possible interference of the GST moiety in adhesion assays. SW-α_9 cells exhibited significant adhesion to the wild type EIIIA segment and six other deletion mutants examined, though the levels of adhesion to each deletion construct varied (Fig. 5*A*). Adhesion to all fragments was inhibited by Y9A2 and reduced to the basal levels observed with the SW-mock cells. Interestingly, two of the shorter deletion mutants, EIIIA24–66 and EIIIA30–57, which include the C-C′ sequence, supported adhesion, albeit at reduced levels, suggesting that the optimal ligand binding site for $\alpha_9\beta_1$ in the EIIIA segment could require both the C-C′ loop and additional flanking sequences. These differences were not due to differential adhesion of the deletion mutant to plastic. Significant SW-α_9 cell adhesion to deletion mutants was also observed when thrombin-cleaved fusion proteins were coupled covalently to plastic using maleic anhydride-activated microtiter plates (data not shown).

MOLT-3 cells that express $\alpha_4\beta_1$, but not $\alpha_9\beta_1$, attached to all five of the EIIIA deletion mutants (Fig. 5*B*). This adhesion was blocked by an α_4-specific mAb, HP2/1 (Fig. 5*B*, *inset*). Similar to $\alpha_9\beta_1$-dependent adhesion, deletion mutants supported less adhesion of MOLT-3 cells than did the wild type EIIIA segment when thrombin-cleaved fusion proteins were either passively (*panel B*) or covalently (maleic anhydride-activated microplates, data not shown) adsorbed to wells. These results suggest that optimal $\alpha_4\beta_1$-mediated adhesion to the EIIIA segment requires the C-C′ loop as well as additional flanking sequences.

Adhesion of MOLT-3 Cells to the EIIIA Segment Stimulates Tyrosine Phosphorylation of p44/42 MAP Kinase—The engagement of integrin α_4 on the surface of THP-1 human monocytic cells with either antibody cross-linking or attachment to a fibronectin substratum has been shown to induce the activation of the p44/42 MAP kinases (48). We hypothesized that the adhesion of MOLT-3 human lymphoblastic leukemia cells to the EIIIA segment would also stimulate the tyrosine phosphorylation of p44/42 MAP kinases. Mn^{2+}-treated MOLT-3 cells were plated onto wells precoated with either an anti-integrin α_4 mAb (HP2/1) or histidine-tagged EIIIA segment. Cell lysates were collected at various time points after adhesion to the EIIIA segment or HP2/1, and tyrosine-phosphorylated p44/42 MAP kinases were analyzed by Western blotting. As shown in Fig. 6, the engagement of integrin $\alpha_4\beta_1$ with the EIIIA segment induced the phosphorylation of p44/42 MAP kinases in a time-dependent pattern comparable to the one shown by the HP2/1-interacted MOLT-3 cells. Total p44/42 MAP kinase was used to normalize the variation of sample loading. The activation of p44/42 MAP kinases in adherent MOLT-3 cells under both conditions was significantly induced after 30 min, peaked after 60 min, and decreased after 90 min of adhesion to the EIIIA segment (Fig. 6, *bottom panel*).

DISCUSSION

We have identified two integrins, $\alpha_9\beta_1$ and $\alpha_4\beta_1$, that bind to the EIIIA segment of FN. Both integrin-EIIIA interactions are blocked by EIIIA-specific mAbs and by their respective anti-integrin mAbs. For both $\alpha_9\beta_1$ and $\alpha_4\beta_1$ the ligand binding sites within EIIIA likely include the C-C′ loop and flanking sequences. Whereas Mn^{2+} is not required for $\alpha_9\beta_1$ interaction with EIIIA, it is required for $\alpha_4\beta_1$-EIIIA interactions. These data identify a novel ligand for both receptors and a new adhesive site within FN that is alternatively spliced. That this alternative splicing is functionally important is indicated by a



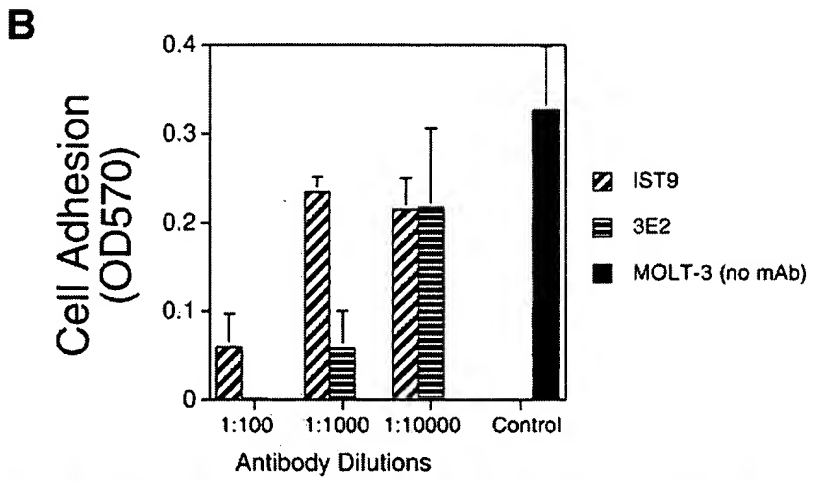


FIG. 3. **EIIIA-specific mAbs block α_9- and α_4-mediated adhesion to EIIIA.** Recombinant EIIIA segment was coated on 96-well microtiter plates at 4 °C overnight. EIIIA-specific mAbs (IST-9 and 3E2) at various dilutions (1:100, 1:1000, and 1:10,000) were preincubated with EIIIA-coated wells at room temperature for 30 min. Unbound antibodies were removed prior to the addition of α_9-transfected SW480 cells (*A*) or MOLT-3 cells (*B*). Cells were incubated at 37 °C for 60 min (SW480) or 90 min (MOLT-3) followed by removal of nonadherent cells by centrifugation. The adhesion of SW-α_9 and mock-transfected SW480 cells (SW-mock) to EIIIA was also determined in the presence (*open bar*, *A*) or absence (*dotted bar*, *A*) of anti-α_9 mAb Y9A2 (10 μg/ml). MOLT-3 cells were pretreated with Mn^{2+} for 30 min at 4 °C prior to plating. Adherent cells were stained with crystal violet and quantified by the measurement of absorbance at 570 nm. Data are shown as the mean (± S.D.) of triplicate measurements from a representative experiment. Experimental conditions are indicated at the *right* of each panel.

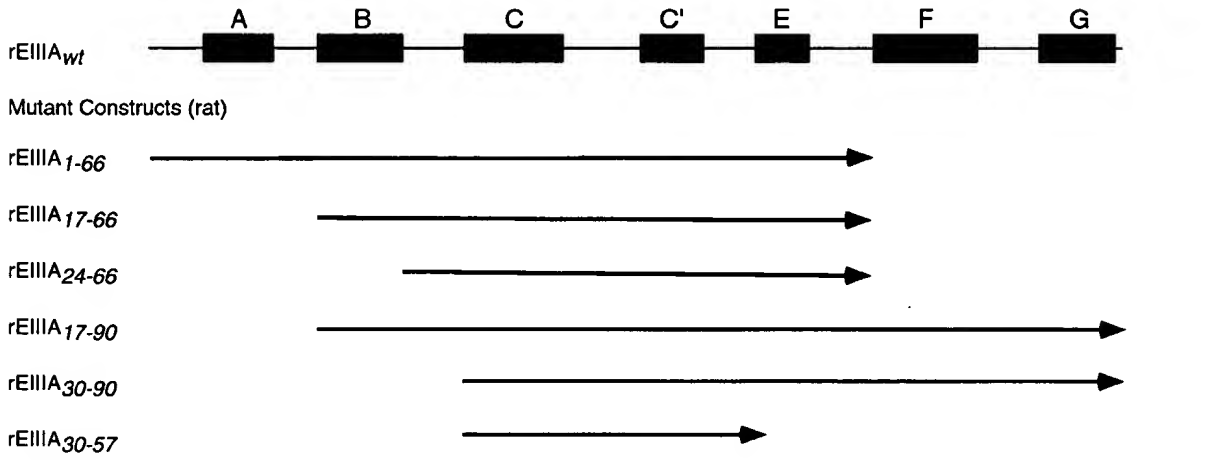


FIG. 4. **Schematic representation of rat EIIIA deletion mutants.** Map of various deletion mutants (*arrows*) derived from wild type rat EIIIA (*rEIIIAwt*, 90 amino acids). Deletion constructs were generated by PCR and subcloned into the pGEX-2T vector as described previously (26). *Arrows* indicate the length of individual deletion constructs relative to the wild type sequence that is shown at the *top* of the figure. *Solid boxes* in the wild type sequence represent the conserved β-strands denoted A, B, C, C', E, F, and G. The amino acids included in truncated mutant rat EIIIA proteins are numbered.

specific increase in EIIIA and $\alpha_4\beta_1$-mediated MAP kinase activation..

Our understanding of EIIIA function has long been hampered by the lack of information on specific cell receptors. The current studies provide the first direct evidence of EIIIA-specific integrins. FN in its most prevalent form, plasma FN which lacks EIIIA, has been recognized as a key ligand for a number of integrins, and its function in mediating cell-ECM (extracellular matrix) interactions has been well established (2). Among these integrins, $\alpha_4\beta_1$ binds to the alternatively spliced CS-1 and CS-5 in the IIICS segment (49, 50), as well as the constitutively present sites H1 in FN-III14 (49) and H2 in FN-III5 (51). Whereas the IIICS segment within plasma FN is a ligand for $\alpha_4\beta_1$, no segment within plasma FN is a ligand for $\alpha_9\beta_1$.

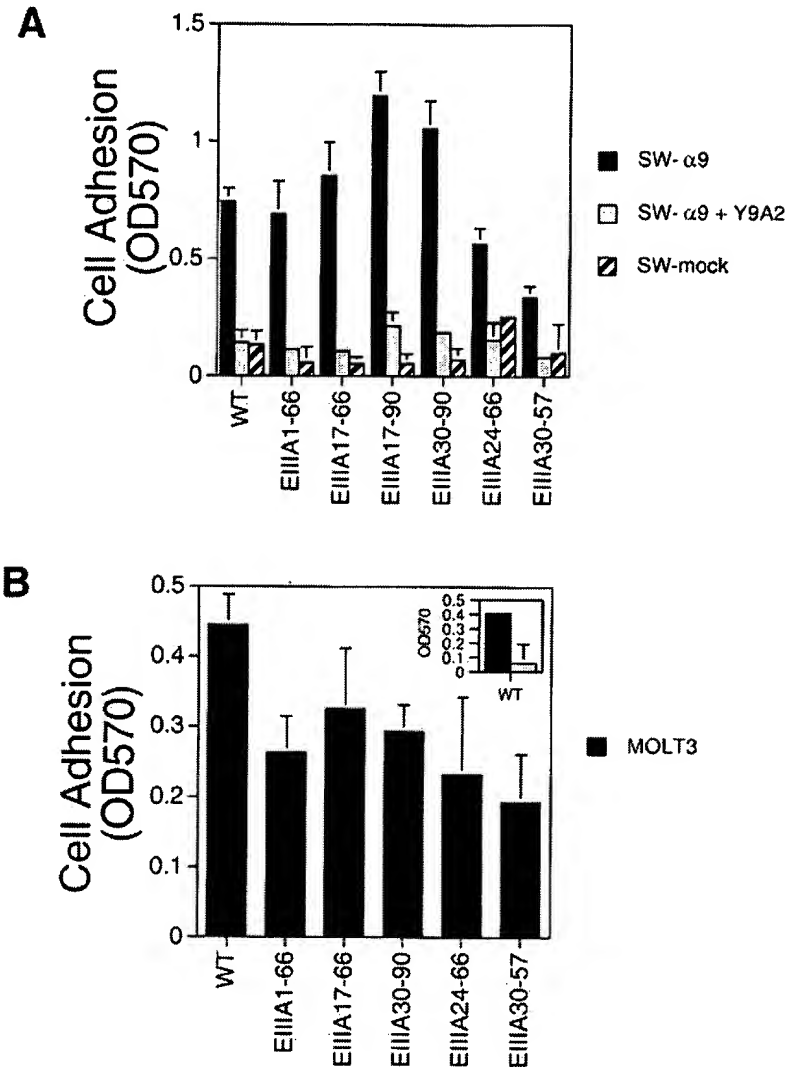


FIG. 5. **The differential adhesion of cells expressing integrin $\alpha_9\beta_1$ or $\alpha_4\beta_1$ to the deletion mutants of the EIIIA segment.** Wild type (*WT*) and deletion mutants (*EIIIA1–66*, *EIIIA17–66*, *EIIIA17–90*, *EIIIA30–90*, *EIIIA24–66*, and *EIIIA30–57*) of the EIIIA segment (1 μM in PBS) were coated on 96-well microtiter plates at 4 °C overnight. The amino acids included in truncated mutant rat EIIIA segment are numbered from their N to C termini as denoted. *A*, transfected SW480 cells were allowed to attach to protein-coated wells at 37 °C for 60 min followed by the removal of nonadherent cells by centrifugation. For blocking experiments, SW-α_9 cells were pretreated with anti-α_9 mAb (Y9A2, 10 μg/ml) for 30 min at 4 °C prior to plating. The adherent SW-α_9 (*solid bar*), Y9A2-treated SW-α_9 (*shaded bar*), and SW-mock (*striped bar*) cells were stained with crystal violet and quantified by the measurement of absorbance at 570 nm. *B*, MOLT-3 cells were pretreated with Mn^{2+} (250 μM) for 30 min at 4 °C. The Mn^{2+}-treated MOLT-3 cells were plated into protein-coated wells followed by incubation at 37 °C for 2 h. Nonadherent cells were removed by centrifugation, and adherent cells were quantified by measurement of absorbance at 570 nm. *Inset* shows that anti-α_4 mAb (HP2/1, 10 μg/ml) blocks the adhesion of the Mn^{2+}-treated MOLT-3 cells to the wild type EIIIA segment (*shaded bar*). Results are expressed as the mean (± S.D.) of triplicate measurements.

Thus, the process of splicing the EIIIA segment into new FN transcripts would generate a novel adhesive motif for $\alpha_9\beta_1$ and an additional site for $\alpha_4\beta_1$. Inclusion of these new sites in EIIIA could underlie the complementary adhesive activity of the EIIIA segment to plasma FN functions (18, 20, 21). Given recent data suggesting a role for EIIIA in cell differentiation, ligation of $\alpha_9\beta_1$ or $\alpha_4\beta_1$ could also signal key changes in cell phenotype without altering cell adhesion.

Expression patterns for $\alpha_9\beta_1$ and $\alpha_4\beta_1$ as well as EIIIA-containing FNs suggest that the interactions of these integrins with EIIIA-containing FNs may subserve different functions. Integrin $\alpha_9\beta_1$ is expressed in adult squamous epithelium, airway epithelium, visceral smooth muscle, skeletal muscle, hepatocytes, and neutrophils (35, 39). During embryogenesis $\alpha_9\beta_1$ is expressed in developing airway, visceral, and vascular smooth muscle at a time closely associated with the appearance of α-smooth muscle cell actin (52). Following vascular injury $\alpha_9\beta_1$ expression is increased in forming neointima.[2] Several of the ligands that bind $\alpha_9\beta_1$, including EIIIA-containing FNs, tenascin, and osteopontin, are also expressed in the neointima (12, 13, 53, 54). Integrin $\alpha_4\beta_1$ is expressed by a narrow spectrum of tissues found predominately in the leukocyte lineage (55). It has been known for many years that most normal adult tissues contain FNs that are largely missing the EIIIA segment (56). However, following injury the expression of EIIIA-FNs is strikingly up-regulated (see Introduction). Recent findings in adult mice depleted of plasma FN demonstrate that plasma FN is not required for normal skin wound healing (57). Importantly, these data suggest a role for locally expressed EIIIA- or EIIIB-variants of FN in healing wounds. Coupling regulated temporal and spatial expression of EIIIA+FNs with ligation by integrin $\alpha_9\beta_1$ or $\alpha_4\beta_1$ could provide a powerful combinatorial approach to generating a regulated response to tissue injury.

The interaction of $\alpha_4\beta_1$ with EIIIA is observed in Mn^{2+}-activated MOLT-3 cells, and $\alpha_4\beta_1$ is the only major integrin on MOLT-3 cells. Our results demonstrating Mn^{2+} dependence are in concordance with a previous report that $\alpha_4\beta_1$-mediated adhesion of leukocytes requires activation of β_1 integrins by divalent cations, stimulatory antibodies, or both (58). It has been shown that 250 μM Mn^{2+} is sufficient to activate $\alpha_4\beta_1$-mediated adhesion of MOLT-3 (37). Mn^{2+} is believed to be a physiological activator of β_1 integrins (45) distinct from other β_1 activators such as phorbol-12-myristate-13-acetate (PMA) and mAb TS2/16. Although the estimated concentration of Mn^{2+} in tissue is 1–14 μM and can be as high as 50 μM in bone or 30 μM in liver (59, 60), higher concentrations of Mn^{2+} (1 mM) have also been used to activate $\alpha_4\beta_1$ (58). The possibility also exists that various concentrations of Mn^{2+} are required for

[2] D. Sheppard, unpublished data.

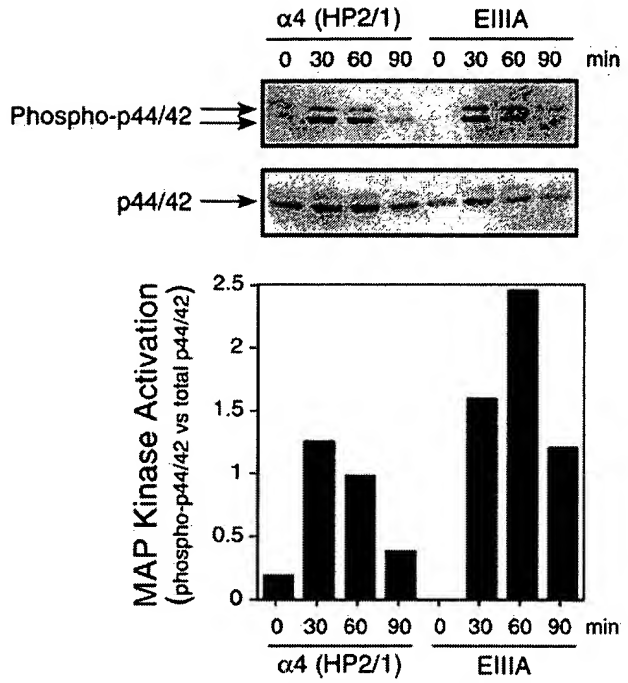


FIG. 6. **Phosphorylation of p44/42 MAP kinases in adherent MOLT-3 cells.** Mn^{2+}-treated MOLT-3 cells (2×10^6 cells/ml) were plated onto wells that were precoated with either anti-integrin α_4 (HP2/1, 2 μg/ml) or histidine-tagged EIIIA segment (100 μg/ml) and blocked with 1% BSA in PBS. Cells were allowed to settle at 4 °C for 30 min before being transferred to 37 °C. After removal of nonadherent cells, lysates of adherent cells were collected at various time points as specified and resolved by SDS-PAGE. Tyrosine-phosphorylated (*Phospho-p44/42, upper panel*) and total (*p44/42, middle panel*) p44/42 MAP kinases were analyzed by Western blotting using anti-phospho-p44/42 MAP kinase polyclonal antibody and anti-p44/42 MAP kinase polyclonal antibody, respectively, as described under "Materials and Methods." The band intensities of phosphorylated p44/42 MAP kinase from different samples were quantified and normalized by the bands of total p44/42 MAP kinase. The activation levels of p44/42 MAP kinase of adherent MOLT-3 cells to HP2/1 or the EIIIA segment at various time points were shown in the *bottom panel*.

$\alpha_4\beta_1$ to achieve optimal adhesion to individual ligands or that the response of $\alpha_4\beta_1$ to Mn^{2+} is cell type-specific. The interaction between $\alpha_4\beta_1$ and the EIIIA segment is likely to be physiologically important because we observe increased phosphorylation of MAP kinase in MOLT-3 cells adherent to EIIIA (Fig. 6).

The current findings provide support for our earlier hypothesis that the epitope within the EIIIA segment for blocking mAbs (*e.g.* IST-9) is in the vicinity of a structural domain of EIIIA, the C-C′ loop, that may be important for EIIIA function. A potential role of the C-C′ loop region in the EIIIA segment for integrin binding has been demonstrated by blocking experiments using EIIIA-specific mAbs including IST-9 and 3E2 (Fig. 3). It is also possible that anti-EIIIA mAbs (*e.g.* IST-9) could inhibit cell attachment indirectly by sterically blocking interactions between cells and some other sequence within EIIIA. One of the deletion mutants of the EIIIA segment (EIIIA30–57) encoding the C-C′ loop and two flanking β-strands (C and C′) exhibits significantly reduced adhesive activities relative to full-length EIIIA (Fig. 5), suggesting that this region is not sufficient to sustain optimal integrin binding. Indeed, our data suggest that in addition to the C-C′ loop, a peptide sequence encompassing amino acid residues 57–66, is also required (Fig. 5*A*). This sequence includes the β-strand E and the E-F loop that is proximal to the C-C′ loop region based on the conserved crystal structure of FN type III repeats (27). Though our data suggest that the C-C′ loop of the EIIIA segment is important to $\alpha_9\beta_1$ and $\alpha_4\beta_1$ integrin binding, it is possible that this region supports adhesion by sustaining an optimal conformation of another ligand binding site.

The appearance of greatly increased levels of EIIIA-containing FN following tissue injury suggests a functional role for the EIIIA segment in wound healing (5). The role of EIIIA in healing wounds and other pathological settings has remained enigmatic for many years. A recent study suggests that either IST-9 or soluble recombinant EIIIA segment can inhibit the TGF-β-induced expression of smooth muscle cell α-actin (α-SMA) in fibroblasts (22). We and others find that $\alpha_4\beta_1$ is a prominent integrin in primary cultured fibroblasts, consistent with the idea that $\alpha_4\beta_1$ could play a functional role during wound healing (61).[3] Therefore it is of interest that we observe (Fig. 6) that the interaction of EIIIA segment with $\alpha_4\beta_1$ in MOLT-3 cells increases phosphorylation of p44/42 MAP kinase. Whereas $\alpha_9\beta_1$ is not present on the fibroblasts that we tested,[3] it is present on neutrophils and keratinocytes. Our current results that $\alpha_9\beta_1$ and $\alpha_4\beta_1$ serve as cell surface receptors for the EIIIA segment suggest novel mechanisms for the regulation of EIIIA-containing FN function during wound healing.

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